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Efficient repair of oxidative damage in mtDNA is essential for survival of the cells undergoing oxidative stress, and may play a role in cancer cells resistance to a radiation therapy. Decreasing mtDNA repair capacity in cancer cells can make them more vulnerable to cancer therapy. Our previous results demonstrated that stable expression of *E.coli* ExonucleaseIII in mitochondria of breast cancer cells diminishes mtDNA repair capacity following oxidative stress, which leads to a decrease in long-term cell survival. Because of the temporary nature of cancer therapy, only transient introduction of proteins into cells is required. In this study we utilize the novel method for direct delivery of purified proteins into cells via protein transduction, combined, for the first time, with targeting of the transduced proteins to mitochondria. Recombinant EGFP and ExonucleaseIII bearing the mitochondrial targeting signal from human Manganese Superoxide Dismutase and protein transduction domain from HIV-1 Tat protein were expressed in *E.coli* and purified. Our results show the high efficiency of the transduction as well as mitochondrial localization of the transduced proteins. Current studies are under way to assess the effect of the protein transduction on ExonucleaseIII activity in the mitochondria and its consequences for mtDNA repair and cell survival.

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INTRODUCTION

Efficient repair of oxidative damage in mitochondrial DNA (mtDNA) is essential for survival of the cells undergoing oxidative stress, and may play a role in cancer cells resistance to a radiation therapy. Decreasing mtDNA repair capacity in cancer cells can make them more vulnerable to cancer therapy, possibly even at a lower dosage. Previous results in our lab demonstrated that stable expression of *E.coli* Exonuclease III in mitochondria of breast cancer cells diminishes mtDNA repair capacity following oxidative stress, which results in decreased long-term cell survival (1). Because of the temporary nature of cancer therapy, only transient introduction of proteins into cells is required. In this study we attempted to utilize the novel method of direct delivery of purified proteins into cells via protein transduction, combined, for the first time, with targeting of the transduced proteins to mitochondria. Recombinant Green Fluorescent Protein (GFP) and Exonuclease III (ExoIII) bearing the mitochondrial targeting signal (MTS) from human Manganese Superoxide Dismutase (MnSOD), and protein transduction domain from HIV-1 Tat protein, were expressed and purified from *E.coli.* We assessed the efficiency of the transduction as well as mitochondrial localization of the transduced proteins. Current studies are under way to assess the effect of the protein transduction on ExoIII activity in the mitochondria and its consequences for mtDNA repair and cell survival.

BODY

We started our experiments with GFP as a test protein, mostly, because it has been an excellent marker for monitoring expression and intracellular localization of a multitude of proteins expressed as GFP fusions. GFP is known to be extremely stable, is not easily denatured, it maintains its fluorescence within wide pH range (pH5.5-12) and it is easily expressed in *E.coli* in large amounts. In our experiments we used a commercially available enhanced version of GFP, EGFP (Clontech). For protein transduction we used an 11 amino acid long Protein Transduction Domain from the HIV-1 TAT protein (TAT-PTD), which, when fused to other proteins, confers to them the ability to be taken up by the cells (2) This process, called protein transduction, does not occur through classical receptor-, transporter-, endosome-, or absorptive-endocytosis mediated processes (3, 4). Proteins containing TAT-PTD, when added exogenously to culture medium, enter the cells in a concentration dependent manner. To target proteins to mitochondria upon entering the cells we used the MTS from MnSOD. The construct used for expressing and purifying recombinant EGFP is outlined in Fig.1. We used oligonucleotides to assemble the synthetic MTS positioned on the N-terminus of the protein, followed by the EGFP sequence (or ExoIII). For the C-terminus of the protein we used another synthetic construct, containing TAT-PTD , and 10 consecutive histidine residues for purification by immobilized metal affinity chromatography. Also, for the detection of recombinant proteins with antibodies,

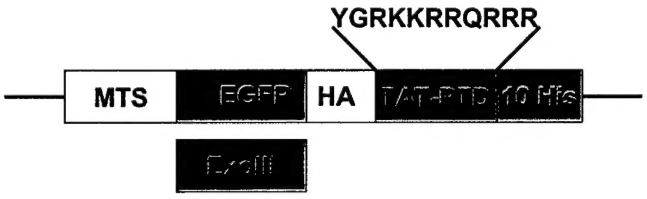


Fig.1 Construct for expression of recombinant proteins in *E.coli.*

hemagglutining tag (HA) was included. Complete fusion construct was made with several rounds of PCR amplification. The PCR products then were cloned and their sequence verified for errors introduced by PCR. The fusion constructs were placed under the control of the inducible *lac* promoter in pET series vectors (Novagen) and introduced in *E.coli* BL21 cells for expression. The cells were grown in liquid cultures, collected by centrifugation and used for subsequent purification. The purification steps are outlined in Fig.2. Since our fusion constructs contain a stretch of 10 histidines, we were able to use affinity chromatography methods to purify fusion proteins from *E.coli* lysates by employing a nickel-NTA agarose column. The bound protein was eluted from column with buffer containing imidazole. The purity of the eluted protein was assessed by SDS-PAGE and Western blot. N-terminal sequencing verified that the purified protein had intact MTS. The similar steps for the expression, purification and identification were used for both fusion constructs: EGFP and, later, ExoIII. The proteins were purified under native conditions.

The proteins will be designated MTS-EGFP-TAT and MTS-ExoIII-TAT in the text.

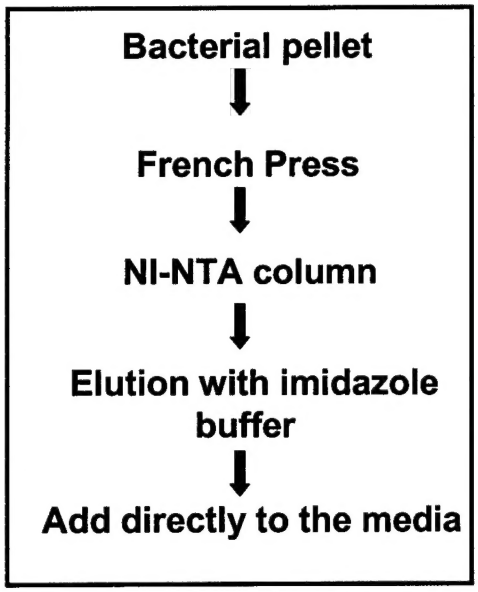
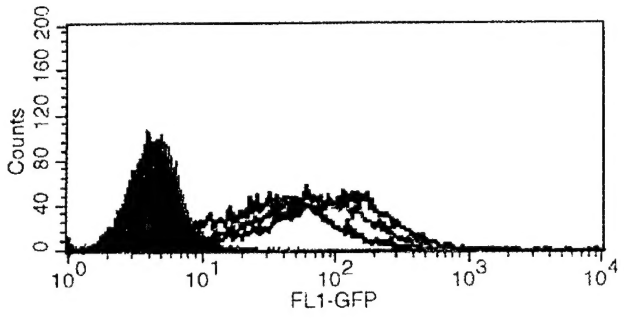


Fig.2 Purification of recombinant proteins from *E.coli* extracts.

For our research we used the MDA-MB-231 human breast adenocarcinoma cell line. Cells were maintained in Eagle's minimal essential media, supplemented with 10% of fetal bovine serum, non-essential amino acids, and L-glutamine. For the transduction experiments we added the purified chimeric MTS-EGFP-TAT directly to the cell media to achieve the concentration in the media 50-80 μg/ml.

To assess the efficiency of the protein transduction process, we used Fluorescence Activated Cell Sorting (FACS) assay (Fig.3). Cells were incubated with the protein in the media for various periods of time (2, 6, and 12 hours), then, cells were trypsinized, collected by centrifugation, and resuspended in PBS. Fig.3 demonstrates the results of FACS analysis. The process of protein transduction was highly efficient: 82% of cells were fluorescent after 2 h of incubation, 92% and 93% were fluorescent after 6h and 12h of incubation respectively.

A.



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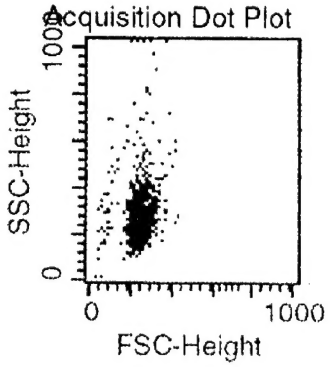

Fig.3 A.-FACS analysis of the of the proportion of fluorescent cells; Cells were incubated with purified recombinant MTS-EGFP-TAT at a concentration of 80 μg/ml of media for indicated periods of time: purple- control, untreated cells; green- 2h of incubation; red- 6h of incubation; blue – 12h of incubation.

B. - Light scattering characteristics of the cells undergoing sorting. Concentration of the signal in the left bottom quadrant indicates that the majority of the cells are intact and healthy.

To asses the intracellular localization of the transduced recombinant MTS-EGFP-TAT protein we used fluorescent microscopy. Cells were treated as indicated in the previous paragraph. After incubation cells were trypsinized and replated on glass coverslips. 12 h later cells were fixed with 4% formaldehyde, mounted on glass slides and observed under fluorescent microscope. Trypsinization and replating steps were necessary to eliminate a strong background made by the excess MTS-EGFP-TAT and also to reassure that the protein was not "stuck" on the outside of the plasma membrane, since trypsin treatment removes most of TAT-fused proteins that has not been taken up by the cell. To visualize mitochondria in the cells for comparison with the fluorescent pattern of MTS-EGFP-TAT, we stained mitochondria in live cells prior to fixation with Mito-Tracker Red CM-H_2 XRos mitochondrion selective dye from Molecular Probes. Fluorescent images were made using Magnafire digital camera and software. Image overlays were made with Image ProPlus v4.1 software.

Fig.4 illustrates the colocalization of MTS-EGFP-TAT fluorescent signal (green) with signal produced by Mitotracker (red) resulting in yellow overlapping areas. From our experiments using different incubation times, we concluded that 12 h of incubation was necessary to allow time for the protein to relocate into mitochondria.

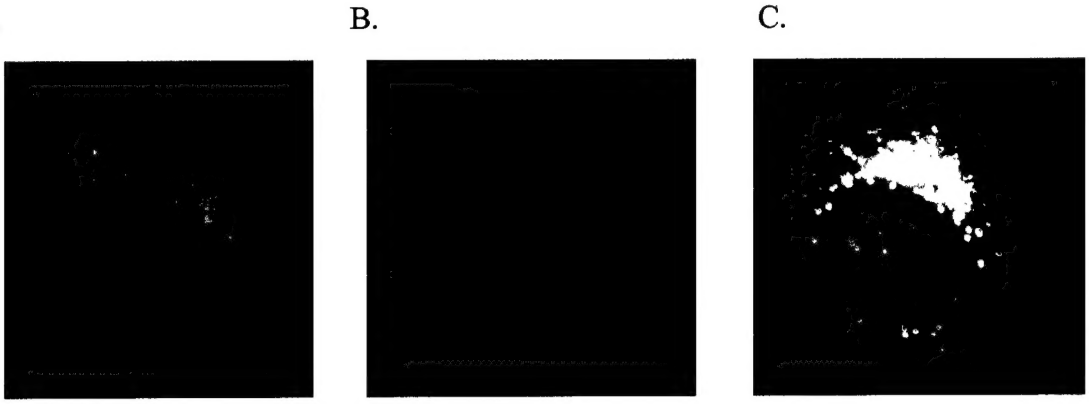

Fig.4 Colocalization of transduced MTS-EGFP-TAT fluorescent signal (A) with Mitotracker mitochondrion selective dye (B), which results in overlapping areas shown in yellow (C).

Our experiments with MTS-EGFP-TAT led us to conclude that we were able to purify from *E.coli* an intact protein, bearing mitochondrial targeting signal and protein transduction domain in pure form, and in large quantities. Also, we confirmed that EGFP, bearing MTS and TAT was, first, efficiently transduced into the cell and, second, targeted to mitochondria.

The next step in our research was the expression, purification and protein transduction of Exonuclease III (MTS-ExoIII-TAT). We used the same protocols for these steps as were developed for MTS-EGFP-TAT and described in the previous paragraphs. We also assessed the catalytic activity of the purified MTS-ExoIII-TAT (Fig.5). [γP^{33}]- end-labeled synthetic 21-mer double–stranded oligonucleotide, containing abasic site at 10^{th} position in one strand was used as a substrate for AP endonuclease and exonuclease activities of ExoIII. Commercially available ExonucleaseIII (Trevigen) was used as a positive control. The products of the reaction were separated in the 20% polyacrylamide- 8M urea gels and radioautographed. The results indicate that we purified an active enzyme. The purified MTS-ExoIII-TAT was stable and retained its catalytic activity for, at least, several cycles of freezing- thawing.

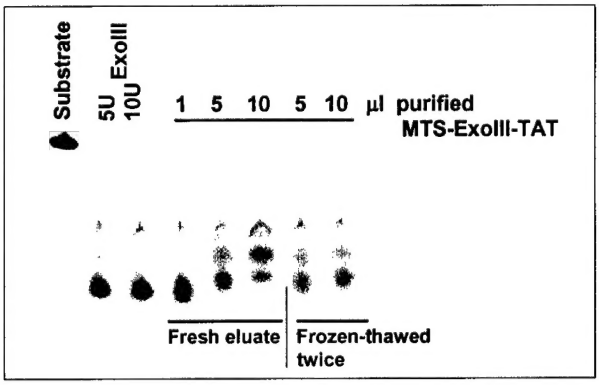

Fig.5 Catalytic activity of purified MTS-ExoIII-TAT;
ExoIII – positive control (Trevigen).

We used the same transduction conditions for MTS-ExoIII-TAT as we used for MTS-EGFP-TAT. The purified MTS-ExoIII-TAT protein was added directly to the cell media to a final concentration of 80 μg/ml. To visualize the internalization and cellular localization of the purified MTS-ExoIII-TAT we stained the fixed and permeabilized cells with monoclonal mouse anti-HA antibodies. FITC-conjugated anti-mouse secondary antibodies were used, and stained cells were observed under the fluorescent microscope (Fig.6). For visualization of mitochondria we used Mitotracker as described for Fig.4.

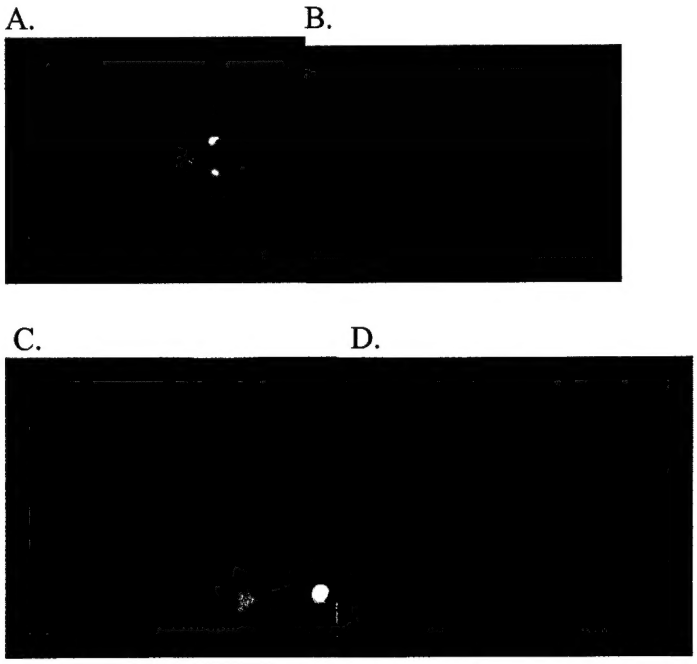

Fig.6 Panels A and C: Staining for HA of the cells incubated with MTS-ExoIII-TAT for 12h. Panels B and D: the same cells stained with Mitotracker mitochondrion selective dye.

In order to verify the mitochondrial localization of the transduced MTS-ExoIII-TAT, we performed Western analysis on mitochondrial fractions isolated from the cells incubated with MTS-ExoIII-TAT for 12 h at a concentration of 80 μg/ml (the same conditions, used in previous experiments). Pure mitochondrial fractions were isolated using method of differential centrifugation, and MTS-ExoIII-TAT was detected in the mitochondrial lysates with anti-HA antibodies (Fig.7). As a positive control we used purified MTS-ExoIII-TAT from the same preparation, that we used for the transduction. Note, that the control protein (Fig.3,lane 3) contains a major band of higher molecular weight, which indicates the unprocessed protein, still possessing the MTS. Upon translocation into mitochondria, the MTS is cleaved off , which would lead to the increase in the appearance of the band with lower molecular weight, which we can see in Fig.3, lane 1.

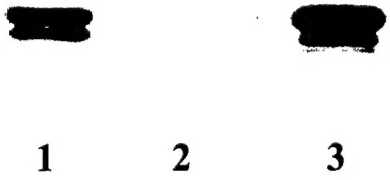

Fig.7 MTS-ExoIII-TAT is detected in mitochondrial fractions of treated cells with anti-HA antibodies. Lane 1- mitochondrial lysates from treated cells; lane 2- mitochondrial lysates from untreated cells; lane 3- purified MTS-ExoIII-TAT used for the transduction experiments.

Research is continuing to evaluate the effect of the transduced ExoIII on mitochondrial DNA repair capacity and long term cell survival following oxidative stress. Preliminary results indicate that cells preincubated with ExoIII for a period of time, sufficient for translocation to mitochondria, show less efficient mtDNA repair after treatment with the superoxide radical generator Xanthine oxidase than control cells.

KEY RESEARCH ACCOMPLISHMENTS

- We expressed and purified from *E.coli* recombinant EGFP and Exonuclease III containing both the protein transduction domain from HIV-1 TAT protein and the Mitochondrial Targeting Signal (MTS) from Manganese Superoxide Dismutase.
- Purified MTS-ExoIII-TAT protein was catalytically active as evidenced by its specific exonuclease activity.
- Both proteins were efficiently transduced into breast cancer cells in culture. The MTS, present on the N-terminal ends of both chimeric proteins directed them to mitochondria.
- This is the first report to our knowledge of using direct protein delivery through protein transduction to target proteins to mitochondria.

REPORTABLE OUTCOMES

These results were presented at the 93rd Annual Meeting of the American Association for Cancer Research, on April 6-10, 2002 in San Francisco, California.
Abstract #3988 "Delivery of green fluorescent protein into breast cancer cells via protein transduction".

Manuscript is in preparation.

CONCLUSIONS

We demonstrated the applicability of the novel method for transient direct delivery via protein transduction of the proteins of potential therapeutic importance into the mitochondria of breast cancer cells. In particular, we delivered purified *E.coli* Exonuclease III into mitochondria.

Our previous data showed that stable expression of Exonuclease III in mitochondria leads to diminished mtDNA repair capacity after oxidative stress. Interference with the normal mtDNA repair process results in the sensitizing cells to oxidative damage. The ability to sensitize cancer cells to therapy involving oxidative DNA damage, such as radiation therapy, would be highly beneficial for the successful cancer treatment. Transient direct mitochondrial delivery of specific proteins, capable of disturbing mtDNA repair under conditions of oxidative stress represents a potential therapeutic strategy for dealing with otherwise therapy resistant cancer cells.

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